The *cta3*$^+$ gene that encodes a cation-transporting P-type ATPase is induced by salt stress under control of the Wis1-Sty1 MAPKK-MAPK cascade in fission yeast

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Received 25 May 1999; received in revised form 20 June 1999

Abstract **In *Schizosaccharomyces pombe*, the Wis1-Sty1 MAP (mitogen-activated protein) kinase signaling cascade is known to play a major role in cellular adaptation to adverse external stimuli, including osmotic stress, oxidative stress, nutrient deprivation, DNA-damaging agents, and heat stress. Nonetheless, it is not known whether or not this particular MAPK cascade is also involved in response to the most common stress, salinity. In this study, we provide evidence that the Wis1-Sty1 MAP cascade is implicated in salt stress response through regulating expression of a salinity-inducible gene. The downstream target gene thus identified is the *cta3*$^+$ gene, which encodes a cation-transporting P-type ATPase. The salt stress-responsive nature of *cta3*$^+$ expression was characterized extensively. It was found that not only the Sty1 MAP kinase but also the Atf1 transcription factor is crucial for the inducible expression of *cta3*$^+$. As far as we know, this is the first instance that the stress-activated Wis1-Sty1 MAPK cascade plays a role in salt stress response in *S. pombe*.**

Key words: Mitogen-activated protein (MAP) signaling pathway; P-type ATPase; Salt stress

1. Introduction

A rapid molecular response is induced in all eukaryotic cells on exposure to adverse environmental conditions. Such a response generally involves changes in the level of gene transcription resulting from intracellular signal transduction in response to a given stimulus. One of such common mechanisms by which eukaryotic cells sense and respond to their external environmental stimuli is via activation of a mitogen-activated protein (MAP) kinase cascade [1–3]. The most widely studied one in mammalian cells is the extracellular signal-regulated protein kinase (ERK) family of kinases [4]. In addition, a new family of MAP kinases that are activated by multiple environmental stresses has been identified. This type of MAP kinase (called stress-activated MAP kinases; SAPKs) is represented by c-Jun N (amino)-terminal kinases (JNKs) and the p38/CSBP enzymes [5,6]. In this regard, recent studies on the fission yeast *Schizosaccharomyces pombe* have begun to provide general insight at the molecular level into both signal transduction and transcriptional regulation in response to a wide variety of external stimuli, because a homologue of SAPK has been identified as Sty1 MAP kinase (also known as Spc1 and Phh1) [7–9]. Importantly, this Sty1 MAPK pathway is activated by a similar range of environmental insults as are the mammalian SAPKs. They include osmotic stress, oxidative stress, nutrient deprivation, UV light, certain DNA-damaging agents, heat stress, and protein synthesis inhibitors [7,8,10–12].

In *S. pombe*, as mentioned above, it is clear that the Sty1 MAPK cascade plays a major role in adaptation to adverse environmental conditions. Hyperosmotic stress results in induction of the *gpd1*$^+$ gene (encoding glycerol 3-phosphate dehydrogenase) [13], oxidative stress results in induction of the *ctt1*$^+$ gene (encoding catalase) [14], heat stress results in induction of the *tps1*$^+$ gene (encoding trehalose 6-phosphate synthase) [11], etc. Interestingly, this cascade somehow integrates stress sensing with control of mitosis, and also links stress signaling with control of sexual differentiation. The common transcription factor (named Atf1) that activates the *gpd1*$^+$, *ctt1*$^+$, and *tps1*$^+$ genes also activates the *ste11*$^+$ gene essential for initiation of meiosis in response to nutrient starvation [15,16]. Thus, the Sty1 cascade not only regulates stress responses but also integrates this response with two processes fundamental to all eukaryotes: control of mitosis and initiation of meiosis. In this regard, for this unicellular microorganism, external salinity must also be a common and adverse environmental stimulus. Nonetheless, such a salt stress response in *S. pombe* has not yet been fully characterized, particularly in terms of the Sty1 MAPK stress sensing network. In this study, we thus wanted to address this particular issue. To this end, a rational way would be first to identify genes whose expression is specifically induced in response to external high salinity, and then, to examine whether or not the expression of such a putative salt-inducible gene is under the control of the Sty1 signaling cascade. Based on this experimental rationale, here we identified a salt-inducible *S. pombe* gene (originally named *cta3*$^+$), which most likely encodes a cation-transporting P-type ATPase (or a cation pump) [17,18]. Furthermore, it was revealed that the salt-dependent induction of *cta3*$^+$ is indeed dependent upon the function of the Sty1 MAPK cascade.

2. Materials and methods

2.1. Strains and media

S. pombe strains, PR109 (*h*$^-$ *leu1 ura4-D18*), JY333 (*h*$^-$ *leu1 ade6-M216*), JY741 (*h*$^-$ *leu1 ura4-D18 ade6-M216*), DW746 (*h*$^+$ *leu1 ura4-D18 ade6-M210 wis1::ura4*$^+$), JM1160 (*h*$^+$ *leu1 ura4-D18 ade6-M216 sty1::ura4*$^+$), and JX305 (*h*$^-$ *leu1 ura4-D18 ade6-M216 atf1::ura4*$^+$) were used [7,16,19]. These strains were grown in YPD or SD medium, supplemented with necessary growth requirements in standard amounts [20].

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Abbreviations: MAP, mitogen-activated protein

0014-5793/99/$20.00
PII: S0014-5793(99)00876-5

2.2. β-Galactosidase assay

Exponentially growing yeast cells in YPD or SD medium were transferred to the same medium containing various amount of solute and incubated for 3 or 6 h. β-Galactosidase activity was measured as described by Ausubel et al. [21].

2.3. Northern hybridization analysis

Northern hybridization analysis was carried out as described previously [22]. Exponentially growing cells in YPD medium were collected and resuspended in fresh YPD medium containing various amounts of solute. A total RNA fraction was prepared from the cells at each time. Hybridization was carried out with probe which specifically encompassing the *cta3*$^+$ coding sequence (2.6 kb *Xho*I-*Hin*dIII fragment indicated in Fig. 1C).

2.4. Gene disruption

The one-step gene disruption method was employed to construct the *cta3* deletion mutant [23]. First, 4241 bp of the DNA region encompassing the *cta3*$^+$ ORF was amplified by PCR using 5′ primer dTTTGCTGCTTATTGTATCTCTAGCC and 3′ primer dTTCTGTGAAAACGATACACTCACGC and cloned at the *Sma*I site of pUC119. For *cta3* disruption, the 1.5 kb *Nde*I-*Nde*I region in the *cta3*$^+$ gene was substituted with the *S. pombe ura4*$^+$ gene (Fig. 1C). Wild type strain JY741 was transformed with the 3.2 kb *Eco*RI-*Hin*dIII fragment of this deletion construct plasmid, then stable Ura4$^+$ transformants were selected. The chromosomal structure of the *cta3* deletion mutant was confirmed by Southern hybridization.

2.5. Construction of a strain carrying a cta3-lacZ$^+$ *fusion gene on the chromosome*

To construct a strain *cta3-lacZ*$^+$ fusion gene on the chromosome, integration vector pHAI202 (Fig. 4A) was constructed as follows. First, a 1.7 kb *Hin*dIII fragment which encodes the *ura4*$^+$ gene was ligated with *Hin*dIII-digested pUC18 to construct pUC(ura4)Hind. Then a 4.8 kb *Bgl*II-*Nco*I fragment which carries the *cta3-lacZ*$^+$ fusion gene was isolated from p11 and ligated in a *Sma*I site of pUC(ura4)Hind to construct pHAI202. Wild type strain JY741 was transformed with pHAI202, and stable Ura4$^+$ transformants were isolated. To confirm that the integration event occurs as described in Fig. 4A, chromosomal DNA was prepared and PCR was carried out using two sets of primers (Fig. 4A). The sequences of PCR primers used were dACATACTCTCTAACTTACTCACCCG (A primer), dCGCCAGGGTTTTCCCAGTCACGAC (B primer), dCAGGAAACAGCTATGAC (C primer), and dTTCTGTGAAAACGATACACTCACGC (D primer). A and D primers correspond to −1863 to −1838 bp and +3171 to +3147 bp, respectively, numbering the translation initiation site of *cta3*$^+$ as +1.

3. Results

3.1. Identification of a salt stress-responsive gene in S. pombe

In the hope of finding genes whose expression is induced under high salinity stress, an *S. pombe* genomic library was constructed with a promoter probing vector (see Fig. 1A, named pYMM5, kindly provided by M. Morimyo). This *Escherichia coli-S. pombe* shuttle vector has multiple cloning sites upstream of the *E. coli* β-galactosidase gene. *S. pombe* genomic DNA segments, digested partially with *Sau*3AI, were ligated into the *Bam*HI site to construct a library. Provided that a DNA fragment carrying a promoter activity was inserted with its correct orientation into the site, *S. pombe* transformants harboring such a recombinant plasmid would give rise to blue colonies on an appropriate agar plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Based on this rationale, *S. pombe* PR109 cells were transformed with the library, and then colonies were replica-plated onto both SD agar plate and SD agar plate supplemented with 0.9 M KCl. It should be noted that these X-gal containing plates were conditioned so as to be neutral (pH 7.0). Of 10^4 transformants, one colony gave a blue color, only if 0.9 M KCl was contained in the SD agar plates (most others were white on both plates, or blue on both plates). The plasmid thus isolated was designated p11. This plasmid clone was expected to carry an *S. pombe* genomic DNA segment that exhibits a promoter activity, whose expression is induced in response to a high concentration of external KCl. To confirm this, the cells carrying p11 were grown for 6 h in liquid medium containing various external solutes, such as 0.9 M KCl, 0.9 M NaCl, 1.6 M sorbitol, and 1.6 M glucose, and then β-galactosidase activities expressed by the respective cells were measured (Fig. 1B). The β-galactosidase activity was markedly induced upon the addition of 0.9 M NaCl, as well as 0.9 M KCl. However, neither sorbitol nor glucose showed such an ability to induce β-galactosidase activities (although a weak induction appeared to occur). These results suggested that the cloned promoter segment responds mainly to salt stress rather than osmotic stress.

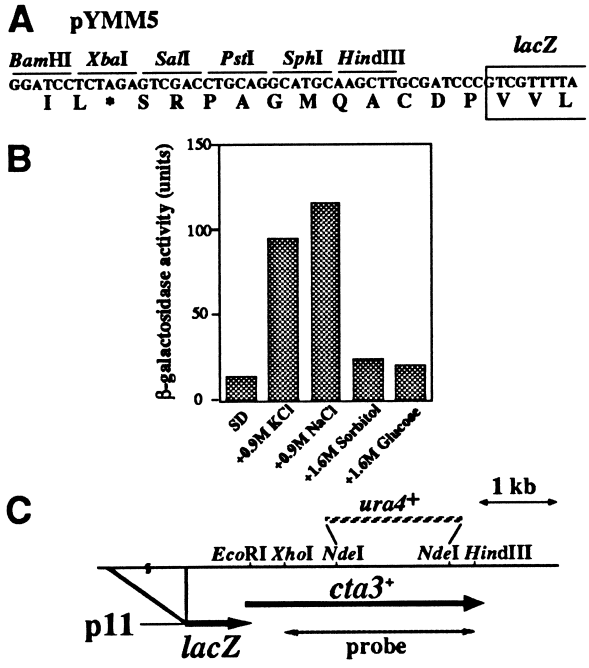

Fig. 1. Structure of plasmid p11 and β-galactosidase activity expressed by p11. A: The nucleotide sequence of the cloning site of promoter probing vector pYMM5 is shown. The amino acid sequences derived from the *lacZ* gene are boxed. B: The wild type *S. pombe* (PR109) carrying p11 was grown in the indicated medium for 6 h and β-galactosidase activities were measured. C: Schematic representation of the *cta3*$^+$ coding region and its upstream region cloned in p11. The probe used for Northern hybridization is indicated.

To identify the gene on p11, nucleotide sequencing was carried out for the cloned segment of about 4 kb. Together with the sequencing result, an inspection of the *S. pombe* genome sequence databases revealed that the segment was derived from the upstream region of the previously identified *S. pombe* gene, namely the *cta3*$^+$ gene (GenBank accession numbers AL021816 and J05634), as schematically shown in Fig. 1C. The 3′ end of the cloned fragment corresponds to the position 852 bp upstream of the deduced translation initiation site of *cta3*$^+$.

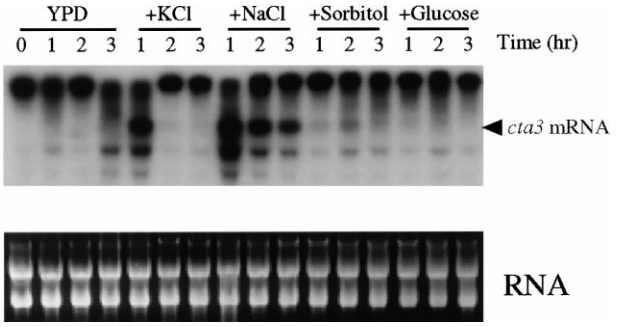

Fig. 2. Northern hybridization analysis using various solutes. Total RNAs were isolated from the wild type cells (JY333), which were grown in either YPD medium (lanes indicated as YPD) or YPD supplemented with 0.9 M KCl (lanes indicated as +KCl), 0.9 M NaCl (lanes indicated as +NaCl), 2 M sorbitol (lanes indicated as +Sorbitol), or 2 M glucose (lanes indicated as +Glucose) for the indicated times (hours), and subjected to Northern hybridization analysis using a probe for the *cta3*$^+$ gene. In the lower panel, the ethidium bromide-stained agarose gel is shown as a control for the amounts of RNA loaded.

3.2. *cta3*$^+$ encoding a P-type ATPase is a salt stress-inducible gene

The *cta3*$^+$ gene has previously been identified as the one encoding a cation-transporting P-type ATPase [17,18]. Although the promoter structure of the *cta3*$^+$ gene has not yet been clarified, as judged from the above result (Fig. 1C), it was assumed that the β-galactosidase activity reflected most likely the promoter activity of *cta3*$^+$. In any case, to confirm this directly, Northern hybridization analysis was carried out using the *cta3*$^+$ coding region as probe (see Fig. 1C), under essentially the same growth conditions as those used for β-galactosidase assays in Fig. 1B. Total RNAs were prepared from the cells grown in YPD medium containing a high concentration of each appropriate solute, and then the *cta3*$^+$ transcript was directly detected (Fig. 2). In the cells grown in YPD medium without any external solute, a very weak signal for the *cta3*$^+$ transcript was detected. Upon the addition of either 0.9 M KCl or 0.9 M NaCl, however, the levels of the transcript in the cells markedly increased within 1 h. It may be noted that the identity of the *cta3*$^+$ transcript (about 4000 nucleotides) was confirmed by analyzing a *cta3* deletion mutant as a reference (data not shown, see below). It should also be noted that the commonly detected top band is most likely due to a contamination of chromosomal DNA segments. In any case, the results of Northern analyses showed that the observed response to KCl was transient, whereas that to NaCl was sustained at least for 3 h, while the addition of either 1.6 M sorbitol or 1.6 M glucose did not give rise to significant accumulation of the *cta3*$^+$ transcript. From these results, we concluded that the *cta3*$^+$ gene is the one whose expression is induced in response to an external high salt stimulus. In this respect, it should be emphasized that the addition of $NaNO_3$ or Na_2SO_4 in the medium also resulted in a marked induction of the *cta3*$^+$ transcript, as in the case of NaCl (data not shown). This result support the idea that cations, like Na^+ or K^+, but not Cl^- anions, are crucial for the induction.

3.3. The *cta3*$^+$ induction is dependent on the Wis1-Sty1 MAPK signal transduction cascade

As mentioned above, we were particularly interested in a salt stress-inducible gene(s), whose expression is dependent upon the Sty1 MAPK cascade. It was thus needed to examine the expression of *cta3*$^+$ with special reference to the underlying signal transduction pathway. In *S. pombe*, it is known that the Sty1 MAPK cascade is linked to an upstream MAP kinase kinase (named Wis1), and to a certain downstream bZIP transcription factor, Atf1 (also known as Gad7), which is directly phosphorylated by the Sty1 MAP kinase [24,25]. Thus, salt stress-inducible *cta3*$^+$ transcription was examined in *wis1*Δ, *sty1*Δ, and *atf1*Δ backgrounds, respectively. As shown in Fig. 3, the induction in response to NaCl stress was largely abolished in all the *wis1*Δ, *sty1*Δ and *atf1*Δ genetic backgrounds tested. These results indicate that the expression of the *cta3*$^+$ gene in response to salt stress is indeed dependent upon the functions of Wis1, Sty1 and Atf1, as anticipated. Essentially the same result was obtained when 0.9 M KCl was used as an alternative salt stress (data not shown). It should be noted that these salt stresses (or osmotic stresses) have already been demonstrated to result in activation (phosphorylation) of the Sty1 MAP kinase [7,8].

With regard to the bZIP transcription factor, Atf1, we do not know whether or not it binds directly to the *cta3*$^+$ promoter region. It is postulated that Atf1 can bind directly to UASs (upstream activating sequence; consensus sequence is TGACGTCA or TTACGTCA) located in various stress-regulated promoters and then trigger gene expression [15,16,25]. Our brief inspection of the *cta3*$^+$ promoter sequence revealed that there exists a putative Atf1 binding sequence (TTACGTAA), located at positions −1105 to −1098, and followed by two putative TATA elements (TATATA), located at positions −973 to −968 and −939 to −934, respectively (the translation initiation codon is taken as +1) (data not shown).

3.4. Construction of a cta3-lacZ$^+$ fusion gene on the chromosome

It became clear that the expression of *cta3*$^+$ was induced by

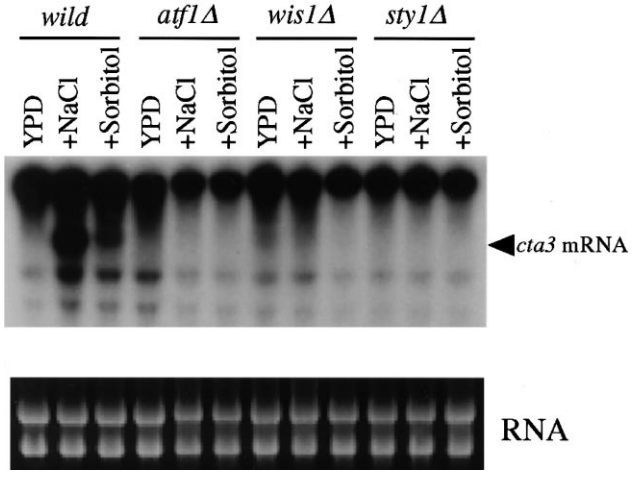

Fig. 3. Northern hybridization analysis using MAP kinase cascade mutants. Total RNAs were isolated from either the wild type (JY333), *atf1*Δ (JX305), *wis1*Δ (DW746), or *sty1*Δ (JM1160) cells, which were grown in either YPD medium (YPD), YPD supplemented with 0.9 M NaCl (+NaCl), or YPD supplemented with 2 M sorbitol (+Sorbitol) for 1 h, and subjected to Northern hybridization analysis using a probe for the *cta3*$^+$ gene. In the lower panel, the ethidium bromide-stained agarose gel is shown as a control for the amounts of RNA loaded.

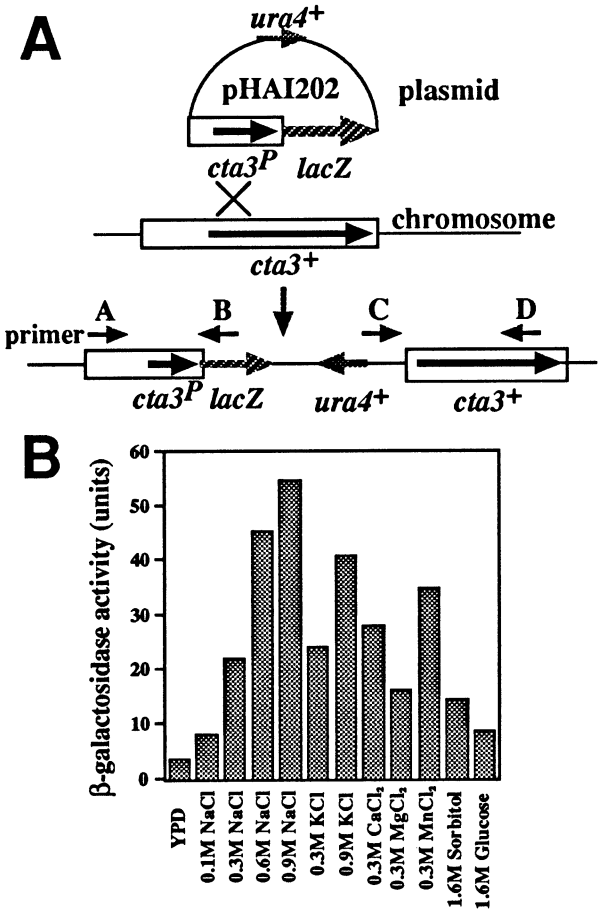

Fig. 4. Schematic representation of integration event for the construction of the *cta3-lacZ*$^+$ fusion gene on the chromosome and β-galactosidase activity expressed by the integrant. A: By a single crossover at the homologous region (indicated X) between plasmid pHAI202 and the chromosome, the *cta3-lacZ*$^+$ fusion gene is constructed on the chromosome as indicated. *cta3*^{P} represents the promoter region of the *cta3*$^+$ gene. Two sets of PCR primers (A and B, C and C) used for confirmation of the chromosome structure are indicated. B: The constructed integrant strain (HAI003) was grown in YPD medium supplemented with indicated concentrations of solute for 3 h and β-galactosidase activity was determined.

an external high salt stress in an Wis-Sty1-Atf1 cascade-dependent manner. Nonetheless, nothing is known about the underlying salt sensing mechanism. To address this issue in future, it may be worth constructing an *S. pombe* strain which will allow us to easily monitor *cta3*$^+$ expression, without adopting any extra plasmid carrying the *cta3-lacZ*$^+$ fusion gene. To this end, we constructed a strain that carries a certain *cta3-lacZ*$^+$ fusion gene on the chromosome (Fig. 4A). Such a strain was constructed by the strategy that the *cta3-lacZ*$^+$ fusion gene was integrated into the *cta3*$^+$ locus on the chromosome through a single cross-over recombination using Ura4$^+$ as a selectable marker. In the resulting strain HAI003, the chromosomal structure of the *cta3*$^+$ locus was verified by polymerase chain reaction (PCR) with a set of appropriate primers (see Fig. 4A). Then, this construct was evaluated in terms of the *cta3*$^+$ induction in response to salt stresses, by monitoring β-galactosidase activities. As expected, β-galactosidase activities in HAI003 fluctuated in response to external KCl and NaCl in a concentration-dependent manner. Note also that such effects of sorbitol and glucose were subtle. These events were consistent with those observed in Figs. 1B and 2. Taking advantage of this strain, we further demonstrated that the addition of external Ca^{2+} or Mn^{2+} divalent cations was also as effective as in the case of monovalent cations. Interestingly, Mg^{2+} was less effective on the induction of *cta3*$^+$expression.

3.5. Characterization of cta3-deficient cells

As mentioned above, the *cta3*$^+$ gene has been identified previously as the one that encodes a cation-transporting P-type ATPase [17,18]. To examine the physiological relevance of our findings in this study, it would be interesting to characterize a *cta3*Δ mutant, particularly with special reference to its sensitivity for growth against external salt stress. Thus, we constructed a *cta3::ura4*$^+$ strain by a standard genetic method. It was then confirmed by Southern hybridization analysis that the resulting haploid strain (named HAI004) indeed carries the *cta3::ura4*$^+$ allele. It was found that HAI004 cells were able to grow on YPD agar plates containing either 0.9 M KCl, 0.5 M NaCl, 0.25 M $CaCl_2$ or 2 M sorbitol (data not shown). It may be worth mentioning that a *cta3*Δ mutant was reported to be sensitive for growth on agar plates containing 0.25 M $CaCl_2$ [17]. We could not confirm this phenotype in our deletion mutant. The reason is not known (it may be due to some difference in growth conditions). Thus, clarification of the physiological function of this putative cation pump must await further intensive examination, particularly in terms of the presumed high salt adaptation in *S. pombe*.

4. Discussion

The Wis1-Sty1 MAPK signaling cascade is known to play a major role in adaptation to adverse external stimuli, including osmotic stress, oxidative stress, nutrient deprivation, DNA-damaging agents, and heat stress [7,8,10–12]. In this study, we demonstrated that this particular MAPK cascade is responsible also for salt stress response, and we further identified its downstream target gene, whose expression is modulated through the signaling pathway in response to salt stress. The identified target gene is *cta3*$^+$, which most likely encodes a cation transporter (or P-type ATPase). The physiological function of the *cta3*$^+$ gene product is not known, as discussed below. However, our findings make sense a priori, if this putative pump is assumed to be involved somehow in a process to extrude or sequester cations from cells. In any event, as far as we know, this is the first instance that the stress-activated Wis1-Sty1 MAPK cascade is implicated also in salt stress response in *S. pombe*.

From the physiological viewpoint, our finding of the salt stress-inducible *cta3*$^+$ gene is interesting, because little is known about the *S. pombe* physiology of adaptive responses to high salinity stress. The *cta3*$^+$ gene was originally isolated and characterized by Ghislain et al., by means of a hybridization method with an appropriate oligonucleotide corresponding to a conserved amino acid sequence among the large family of P-type ATPases [17]. They reported that Cta3-deficient cells result in an accumulation of a higher level of cytosolic free Ca^{2+}, presumably due to a reduction of Ca^{2+} uptake into intracellular compartments. It was also noted that such a *cta3*Δ mutant exhibits sensitivity for growth on agar plates containing 0.25 M $CaCl_2$ [17]. We could not confirm this

phenotype in our deletion mutant. The reason is not known (it may be due to some difference in growth conditions). Furthermore, they did not address the issue of the transcriptional regulation of this gene. Here we found that the inducible nature of *cta3*$^+$ expression is not specific toward Ca^{2+}, but rather, its transcription is triggered by general salt stress. Thus, *cta3*$^+$ may be implicated in a global response to external salt stress. Nevertheless, so far, we have not observed any growth defect in our *cta3Δ* mutant, particularly in terms of sensitivity to high salt stress. In this regard, it may be worth mentioning that, in *S. pombe*, the *sod2*$^+$ gene that encodes a Na^+/H^+ antiporter was reported previously to be involved in tolerance of cells to external Na^+ and Li^+ by pumping out these toxic monocations across the plasma membrane [26]. It can thus be reasonably assumed that *S. pombe* may have redundant ion transport systems that operate against external salt stress. In any event, clarification of any defect of *cta3*Δ cells must await further characterization.

It should be mentioned here that the adaptation mechanisms toward salinity stress has been extensively characterized in the budding yeast *Saccharomyces cerevisiae* [27,28]. The best characterized is the *ENA1/PMR2A* gene that encodes a P-type ATPase involved in extrusion of Na^+ and Li^+ from the cytoplasm [29,30]. *ENA1/PMR2A* transcription is induced by salt stress, and interestingly, this induction was dependent upon both the calcineurin pathway and the high-osmolarity glycerol (HOG) MAP kinase pathway [31,32]. Our findings in this study may be reminiscent of the above scenario in the budding yeast. As emphasized previously, however, the *S. pombe* Sty1 cascade is quite distinct from the *S. cerevisiae* HOG1 cascade from several viewpoints [33]. Furthermore, it should be emphasized that, not only in *S. pombe*, but also in *S. cerevisiae*, the following crucial and general issue remains to be addressed.

In *S. pombe*, the underlying molecular mechanisms, by which the single Wis1-Sty1 cascade can manage a wide variety of multiple external stimuli, are entirely elusive. *S. pombe* cells may have respective signal perception systems, each of which is responsible for each specific stimulus. Signals thus propagated may somehow converge (or be integrated) into the main Wis1-Sty1 MAPK cascade. Even so, this mechanism has its own dilemma, because the Wis1-Sty1 MAPK cascade has to provide each specific output (or consequence), depending upon the external stimuli treated. This paradoxical issue is a long-standing subject of debate in general, and such a problem can be seen in our findings. Provided that *S. pombe* cells were treated with 2 M sorbitol (a non-ionic osmotic solute), the Wis-Sty1 cascade together with the downstream Atf1 transcription factor must demand the activation of *gpd1*$^+$, but not that of *cta3*$^+$ (see Fig. 2 and Section 1). On the other hand, if the same cells were treated with 0.9 M NaCl (a salinity and osmotic solute), the activation of both the *gpd1*$^+$ and *cta3*$^+$ genes should be ensured through the same Wis1-Sty1-Atf1 signaling pathway. The molecular logic behind such a sophisticated signaling network is entirely elusive. In this respect, comparable studies on the signal transduction systems of the Wis1-Sty1-Atf1-Gpd1 and Wis1-Sty1-Atf1-Cta3 pathways will provide a paradigm to address experimentally these general issues mentioned above. This is in fact our motivation to have begun this approach in *S. pombe*, and thus relevant lines of experimentation are under way.

Acknowledgements: The authors are grateful to M. Morimyo (National Institute of Radiological Sciences, Japan) for plasmid pYMM5. This study was supported by grants from the Ministry of Education, Science and Culture of Japan.

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